

SAFETY AND PROTECTIVE EFFICACY OF RESPIRATORY SYNCYTIAL VIRUS-LIKE
PARTICLES (RSV VLPS) IN A MURINE MODEL

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ABSTRACT

Respiratory syncytial virus (RSV) is the leading cause of severe respiratory infections in children worldwide, particularly bronchiolitis and pneumonia. Most children will have been infected with RSV by the time they reach the age of 2, with incidence rates higher during the early months of infancy. It is estimated that in the United States, an average of about 58,000 children under the age of 5 are hospitalized due to RSV each year. To this day, there is still no licensed vaccine. In this study, we evaluated the safety and efficacy of monophosphoryl lipid A (MPLA)-adjuvanted RSV virus-like particles (RSV VLPs) that express the F and G surface proteins and the M matrix protein, in a murine model. F is a crucial protein in RSV vaccine development because it contains neutralizing epitopes that can be targeted by neutralizing antibodies. We conducted an *in vivo* analysis by immunizing BALB/c mice with diluent, RSV, MPLA-adjuvanted RSV VLPs, or non-adjuvanted RSV VLPs. Our results indicate that two intramuscular immunizations of the MPLA-adjuvanted RSV VLPs elicited a strong neutralizing antibody response. It also showed that immunization induces a RSV-specific IgG response and higher IgG2a levels compared to IgG1 which suggests a Th1-biased response. MPLA-adjuvanted RSV VLPs was also able to protect mice from RSV infection in the upper and lower respiratory tracts and the development of disease in the lungs based on the histopathology of the lungs. Our findings thus far lead us to conclude that using MPLA-adjuvanted RSV VLPs is a promising candidate for a RSV vaccine.

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CHAPTER 1

INTRODUCTION

Paramyxoviruses

The *Paramyxoviridae* family includes a variety of infectious agents such as the measles virus, mumps virus, parainfluenza viruses (PIV), Nipah virus (NiV), and respiratory syncytial virus (RSV). This family is further classified into two subfamilies: *Paramyxovirinae* and *Pneumovirinae*. The *Paramyxovirinae* subfamily contains seven genera (*Respirovirus*, *Rubulavirus*, *Mobillivirus*, *Henipavirus*, *Aquaparamyxovirus*, *Avulavirus*, and *Ferlavirus*) while the *Pneumovirinae* subfamily is composed of two genera (*Pneumovirus* and *Metapneumovirus*). Paramyxoviruses are enveloped negative-sense RNA viruses, characterized by the presence of the fusion (F) protein which is responsible for viral fusion with the host cell [24].

Respiratory Syncytial Virus (RSV)

Human respiratory syncytial virus (RSV) is from the *Pneumovirinae* subfamily and *Pneumovirus* genus of the *Paramyxoviridae* family [3, 60]. There are two RSV subgroups: A and B. RSV was first isolated in 1955 from a laboratory chimpanzee that showed signs of common cold-like infection. Subsequently, it has been isolated from infants who suffer from respiratory illness [24]. It is currently the most common cause of pediatric acute lower respiratory infection (ALRI) worldwide, but can infect humans across all ages as well as cause severe disease in high risk populations such as the elderly and immunosuppressed individuals [60]. Although RSV is one of the leading viral agents that is associated with ALRI, leading to pneumonia and bronchiolitis, there are still no RSV-specific antivirals or vaccines available.

Genome

RSV has a linear negative-sense single-stranded RNA genome, approximately 15 kb in length (Fig.1A). The genome contains 10 genes that encode for 11 proteins: nonstructural proteins NS1 and NS2; polymerase complex proteins P (phosphoprotein), N (nucleoprotein), and L (polymerase); M (matrix protein); surface proteins SH (small hydrophobic protein), G (glycoprotein), and F (fusion protein); and transcription and replication modulators M2-1 and M2-2 [27, 52, 74, 83]. The genome is not capped or polyadenylated, but tightly bound to the N protein, thus creating a nucleocapsid resistant to RNase degradation. This encapsidation further allows the virus to more effectively evade the immune system by protecting it from recognition by host cell pattern recognition receptors (PRRs) [24].

Virion Structure

RSV is a pleiomorphic virus that can either be regular- or irregularly-shaped spheres, approximately 100 to 350 nm in diameter, or filamentous particles, about 60 to 200 nm in diameter and up to 10 µm in length (Fig. 1B-C) [24]. The viral particle is composed of a nucleocapsid within a lipid envelope. The RSV envelope expresses three transmembrane viral proteins F, G, and SH, as well as a thin layer of the M protein on the inner surface of the envelope. The RSV genome is closely associated with four of the viral proteins: N, P, M2-1, and L [24, 43].

Replication Cycle

The RSV replication cycle (Fig. 2) begins when the viral envelope fuses with the host cell membrane through the interaction of viral envelope proteins G and F with host cell receptor molecules such as heparin sulfate and chondroitin sulfate B. Next, the

encapsidated genome and RNA-dependent RNA polymerase (RdRp) are released into the cytoplasm where viral protein synthesis occurs. RSV is known to exhibit a polar transcription gradient due to the tendency of the RdRp to fall off the genome mid-transcription and start over. Because of this, there are higher mRNA levels of promoter-proximal genes. The negative sense genome is used as a template to generate the antigenome, which is essentially the positive sense strand. The antigenome is also encapsidated and used as a template to produce more copies of the negative sense genome, leading to more viral protein production, thus perpetuating the cycle of antigenome → genome → viral protein production. To exit the cell, a copy of the negative sense genome and viral proteins migrate toward the plasma membrane. The newly synthesized virus buds out of the host cell through a lipid raft expressing viral envelope proteins [3, 10, 14, 24, 58].

F Protein

RSV F is a trimeric type I transmembrane protein within the viral envelope. Of the 11 RSV proteins, the F and G proteins are considered to be the most immunogenic. However, the F protein is of particular importance for two reasons: (1) it mediates viral fusion with the host cell and (2) its F1 subunit contains all the neutralizing epitopes that can potentially be targeted by drugs and vaccines. The F monomers are referred to as F0 (about 67 kDa) when they are initially synthesized, but as they mature they are cleaved by a furin-like protease into the F1 and F2 polypeptide fragments, which are linked by disulfide bonds [24, 40, 69, 72]. During the later stages of RSV infection, the mature F protein is expressed on the membrane of the infected host cell in a pretriggered form, allowing it to fuse with neighboring cells and form large multinucleated cells called syncytia. Once the

F protein recognizes a host cell receptor, it is triggered and undergoes an irreversible conformational change activating a mechanism that forces the viral and host cell membranes to fuse together [11, 50].

Epidemiology

RSV is a highly infectious pathogen that can cause upper and lower respiratory infections. It is the most common cause of pediatric lower respiratory tract disease worldwide and is second to influenza in causing medically significant respiratory tract disease in adults [24, 52]. It has been estimated that RSV accounts for over 33 million new acute lower respiratory infection cases worldwide each year in children under the age of 5 and at least 3.4 million hospitalizations due to severe disease. Global mortality as of 2010 was estimated at over 250,000 deaths however, this number may be an extreme underestimation due to under-reporting especially in developing countries [82]. In the United States, it has been estimated that there are about 125,000 hospitalizations, approximately half of which are children under the age of 5, and 250 infant deaths each year due to RSV infection [9, 60]. Nearly all children will have been infected with RSV by the time they are 2 to 3 years old, with incidence rates higher at 1-3 months [51, 60, 74]. Primary infection is almost always symptomatic with 25% to 40% of primary infection cases resulting in lower respiratory tract infection [24]. Furthermore, mortality and morbidity rates for premature infants and infants with chronic lung or congenital heart disease are higher. Reinfection can occur at any time, especially during the first few years of life and with increased exposure, and tends to be symptomatic. Although RSV infection is symptomatic, subsequent infections do not normally lead to as severe a disease as primary infection. Major risk factors for RSV infection include exposure to high risk

environments such as daycare centers and hospitals, low maternal antibody titers acquired *in utero*, premature birth, comorbidities (chronic cardiac or pulmonary disease, immunodeficiency, etc.), family history of pulmonary disease, and exposure to cigarette smoking [24, 43, 64, 82]. There is seasonality associated with RSV infection, with typical seasonal RSV infections occurring in the winter and spring months in temperate climates. However, duration and length of season can vary by region. RSV is easily transmissible through direct person-to-person contact and self-inoculation after touching contaminated objects then touching mucosal surfaces such as the eyes, nose, and mouth. Nosocomial infections are also possible, especially during the RSV season [9, 51, 74].

Clinical Features

RSV symptoms are very similar to those of the common cold and are predominantly associated with upper respiratory infection in healthy children and adults. The incubation period is about 4 to 6 days, but it can range from 2 to 8 days [24, 27, 39].

RSV exhibits tropism for the ciliated cells of the nasopharynx and continues moving down to the lower respiratory tract where it can infect alveolar cells, thus resulting in inflammation, thickening of interalveolar walls, lymphocytic infiltration, and cell death [14, 19, 43, 58, 60]. In most cases, patients can recover after several days to a few weeks of illness. Symptoms include a runny nose, fever, cough, congestion, and sore throat. However, in high risk children and adults the lower respiratory tract may be involved [9, 19]. The most common lower respiratory diseases caused by RSV infection are bronchiolitis and pneumonia, which are associated with airway obstruction due to inflammation, mucus hypersecretion, and shedding of necrotic cells in the bronchioles and alveoli [43]. Full-term infants do not usually develop severe ALRI due to larger, fully

developed lungs, as well as the presence of maternally-acquired antibodies. Preterm infants, on the other hand, have smaller lungs and are born without receiving the maximum levels of maternal antibodies so they are at higher risk for ALRI upon RSV infection [24, 43]. Although still under investigation, some studies also indicate that severe lower respiratory disease due to RSV infection in infants and young children can lead to abnormal pulmonary function (e.g. asthma, recurrent coughing and wheezing) for many years after the initial infection [24].

Immune Response

The human immune response to RSV is not definitively understood. Ethical and practical issues associated with studying RSV in the infant target population further hinder studies on the human immune response to RSV [43]. RSV infection is generally resolved within a few weeks, but natural infection in children and adults does not confer long-lasting protection which is why reinfection is so common. Upon infection, neutralizing antibody (NtAb) and cell-mediated responses are usually induced. However, the immunological mechanisms surrounding the failure of natural RSV infection to confer protection are still unclear. The inability to establish an effective memory response and the diminishing RSV-specific NtAb antibody titers over time might be to blame [16, 27, 43]. Previous animal studies have shown that F and G are the main virus neutralization antigens. Vaccines and therapeutics that target these antigens might have the potential to confer long-lasting protection [19, 24].

Innate Immune Response

Innate immunity plays a crucial role in defending the host against RSV infection. Physical barriers such as the mucosal lining of the nasopharynx and ciliary sweeping aid in restricting infection. Pulmonary surfactant proteins (SPs) are part of the innate mechanisms utilized against RSV infection and aid in innate responses such as opsonization and complement. SP-A, for example, can bind to the F protein and help neutralize RSV [51]. *In vitro* and murine studies have shown that SPs might have the ability to neutralize RSV by binding to the F protein and aid in viral clearance [26, 27]. In a study by Kerr et al., it was also observed that there was a decrease in pulmonary SP concentrations from bronchoalveolar lavage (BAL) samples collected from infants with severe RSV-related respiratory disease [38].

Toll-like receptors (TLRs), particularly TLR4, expressed by a variety of cells are also important in the innate immune response to RSV. Studies using TLR4-deficient mice have shown that TLR4 can help mediate the innate response to the F protein and a lack of TLR4 can severely impair NK cell function, IL-12 expression, and the ability to clear the virus [28, 40, 41]. Despite the benefits of stimulating TLRs upon RSV infection, in excess it can contribute to pathogenesis through the overstimulation of TLRs and overproduction of pro-inflammatory cytokines. One of the major consequences of excess pro-inflammatory cytokines is the unrestricted recruitment of immune cells (e.g. neutrophils, eosinophils, lymphocytes, mononuclear cells) to sites of infection [14, 24, 43, 51]. From past studies in which infant upper and lower respiratory tract washes were collected, it was found that RSV-infected subjects had a greater number of immune cells in their washes [21]. Because all these immune cells have the potential to produce more pro-

inflammatory cytokines, an excess of these cells can further activate the inflammatory response, thus causing pathology.

It is important to keep in mind that when the inflammatory response is properly regulated, it can efficiently control RSV infection. Murine studies, for example, have shown that NK cells are essential in RSV clearance due to their ability to produce IFN- γ , destroy infected cells, and contribute to the Th1 response [24, 43]. The granulocytic activity of neutrophils and eosinophils have also been shown to restrict the virus and have been detected in respiratory washes of infants with severe RSV disease [21]. Neutrophils, in particular, have the ability to produce IL-9, which can promote mucus production and aid in restricting infection [47]. Macrophages are some of the first cells that encounter the virus and might also play an important role in restricting RSV, especially during the early stages of infection, by producing pro-inflammatory cytokines and clearing any debris that can further heighten the inflammatory response [24, 51].

T Cell-Mediated Immune Response

Studies comparing RSV clearance between immunocompetent children and those with deficient cellular immunity suggest that cell-mediated immunity is also important in restricting RSV infection [51]. The CD8⁺ T cell response to RSV is still not well-understood. CD8⁺ T cells are well-known for their cytotoxic ability and previous murine model studies indicate that they may play a role in clearing RSV infection, however, they may also contribute to pathology [19, 27, 51]. In infants, it has been shown that those with severe RSV disease have increased T lymphocyte levels in the respiratory tract, with a greater proportion of them being CD8⁺. This has led researchers to believe that CD8⁺ T cells might contribute to pathogenesis. Upon further investigation, however, although

there was a higher proportion of CD8+ lymphocytes, they did not make up the most abundant cell population in samples retrieved from infants with severe RSV disease [21]. Another study comparing the peripheral blood mononuclear cells (PBMCs) of RSV-infected infants with controls showed that CTL activity was at its peak within 1 week of infection and was also age-dependent with about 35-38% of infants under 5 months of age and more than 65% of 6- to 24-month olds showing CTL activity against RSV [12]. Meanwhile a study that looked at serum CTL and cytokine responses in infants followed for 3 consecutive RSV seasons showed that a high percentage of the infants developed RSV-specific CTL activity. Although short-lived, CTL response could be enhanced after subsequent infections [51]. The CD4+ T cell response might also play an important role in RSV-specific immunity and must be taken into consideration when developing a RSV vaccine due to the Th1/Th2 polarization sometimes seen in patients with enhanced respiratory disease (ERD). There is evidence from murine studies that a Th2-biased response against RSV can contribute to pathogenesis because it can lead to the production of cytokines that are associated with ERD (e.g. IL-4, IL-5, and IL-13) [36]. Meanwhile, a Th1 response can lead to the production of cytokines associated with viral clearance (e.g. IFN- γ and IL-2). Animal studies have also shown that inducing a balanced Th1 response can improve the host response to RSV infection [17, 28]. However it is still unclear whether RSV infection directly causes a shift in T helper cell polarization in humans. Some studies using *in vitro*-stimulated PBMCs collected from infants with RSV disease showed that cells from RSV-infected infants produced more IL-4 than IFN- γ , while other studies showed a Th1- or a Th1/Th2- mixed response indicating that there is

some variability in infants' ability to produce a Th1, Th2, or heterogeneous response [4, 24, 45].

The Th1 and Th2 responses to RSV are often studied because of their close association with ERD. However, the role of other T helper cell populations, such as regulatory T cells (Treg) and Th17, have been investigated but so far limited to murine studies. A murine study by Shao et al. [65] demonstrated that Tregs play a role in modulating B cell activity, particularly the production of RSV-specific NtAbs, and depleting Treg populations can lead to low NtAb titers, thus exacerbating respiratory disease. Th17 is often associated with inflammatory and autoimmune responses and tends to have the opposite effect as Tregs during RSV infection. Studies have shown that a strong Th17 response can lead to an increase in inflammation, suppression of CD8 T cells, and increased mucus production in murine models [8].

Antibody Response

The infected host is capable of mounting an antibody response to RSV infection and there are many studies, both animal model and human, which suggest that the presence of antibodies can be protective. Mouse studies have shown that mice incapable of producing antibodies against RSV exhibit more severe illness and lung pathology [68]. Infants are also born with some protection, having acquired RSV-specific serum IgG from the mother through the placenta starting at approximately 26 weeks gestation, and can continue to receive antibodies from the colostrum through breastfeeding [24, 43]. Although these titers diminish over time at a half-life of about 21 to 26 days, and the lower titers achieved in premature infants put them at higher risk, previous studies indicate that infants born

with higher RSV antibody titers develop milder disease or are infected at an older age [24].

NtAbs are key correlates of protection for most paramyxoviruses, including RSV [77]. Humans have the potential to produce antibodies to most RSV proteins, but the F and G proteins tend to generate the most potent NtAb response [51]. These NtAbs are capable of binding to the RSV surface proteins (primarily the F protein) and inhibit its ability to mediate viral fusion to host cells. In various RSV studies, inducing a sufficient NtAb response is considered an essential characteristic of a safe and effective vaccine or treatment.

The secretory IgA response is also thought to be important in restricting infection, especially in the upper respiratory tract. Studies by Valosky et al. [75] and Sharma et al. [66] showed that infecting mice with live RSV enhanced the production of IgA. In humans, IgA has been detected in nasal secretions of RSV-infected infants [49]. Although the IgA titers wane over time, the response has been known to become more sustained as the individual grows older and is subsequently reinfected [76].

The significance of IgE production is still under investigation, with a few murine studies suggesting that it contributes to RSV-related pathology because IgE production is induced by Th2 cytokines such as IL-4 [15]. Young children with acute bronchiolitis due to RSV infection have also been found to exhibit high levels of IgE [71]. Despite these findings, there is still not enough evidence supporting such an association so the relationship between IgE and RSV is still a topic that requires further research [24, 51].

Memory Response

It is still unclear why natural infection with RSV fails to confer long-lasting protection and allows reinfection in humans. There could be a variety of factors that contribute to the frequency of reinfection such as the host immune response, infection history, or the circulating RSV strain during a given season, to name a few [43]. Although the memory response is not well-understood, murine and human studies do show that there is a positive correlation between the presence of memory CD8 T cells and susceptibility to RSV disease. In a murine study by Olson et al., it was found that having a high number of RSV epitope-specific memory CD8 T cells can inhibit the development of pulmonary eosinophilia and the production of Th2-associated cytokines [57]. Meanwhile, in a human study comparing elderly patients with chronic obstructive pulmonary disorder (COPD) with healthy patients, it was found that low RSV-specific memory CD8 T cell numbers in elderly patients with or without COPD can contribute to increased susceptibility to RSV infection [16].

Diagnosis and Treatment

A clinical diagnosis of RSV infection is often difficult due to its clinical similarities to other respiratory viral pathogens such as parainfluenza viruses, influenza virus, rhinovirus, and coronavirus [3, 52]. There are also a number of bacterial agents that are associated with similar symptoms to RSV infection. Different patient samples can be used for diagnosis such as sputum, bronchoalveolar lavage, nasopharyngeal swabs, and nasal washes. However, there could be some variation in the ability of diagnostic methods to detect the virus depending on the patient sample type. For example, some studies suggest that nasopharyngeal swabs yield lower viral titers compared to nasal washes or sputum are better samples when using RT-PCR [22, 52, 67]. Laboratory test culture is the best way

to diagnose RSV, with virus isolation from patient samples as the gold standard. However, this method takes time so more rapid techniques are used in which viral antigens in nasal swabs and secretions are detected [24, 52]. A lateral flow immunochromatography-based rapid antigen test may be the most clinically relevant diagnostic method nowadays and takes approximately 30 minutes, but has much lower sensitivity so negative results must be confirmed with other diagnostic tools. There are various lateral flow immunochromatography-based RSV tests such as Binax Now RSV, QuickLab RSV Test, and RSV Respi-Strip [3, 26]. In a research setting, other diagnostic methods are used to more accurately detect the presence of RSV in samples. Direct immunofluorescence assays (DFA) and enzyme-linked immunoassays (EIA) are frequently used to detect RSV antigens, primarily the F protein, while techniques such as real-time PCR can detect RSV RNA expression. [3, 52].

Treatment for RSV infection is based on symptomatic intervention and supportive care because there are no RSV-specific treatments or vaccines available. There are, however, drugs to help alleviate the cold-like symptoms associated with RSV such as over the counter decongestants, antihistamines, and pain and fever reducers [3, 52]. Most patients will develop mild illness and recover on their own with plenty of rest, hydration, and use of over the counter medicine to relieve symptoms [60]. Patients with severe lower respiratory infection, however, must be kept under observation and receive supportive care when necessary. This includes mechanical removal of excess mucus, repositioning of the patient to help their breathing, providing intravenous fluids, and providing respiratory assistance using a ventilator [24, 60]. There are also anti-inflammatory approaches that have been used to care for patients with severe RSV disease.

Corticosteroids and inhaled bronchodilators, for example, have been used for RSV therapy. However, such treatments provided little to no improvement [46, 60, 74]. Ribavirin, a broad spectrum antiviral, was approved in 1986 to be used as RSV treatment in the United States. Challenges in aerosolizing ribavirin, some of its side effects, and studies indicating that it does not result in dramatic respiratory improvement keeps it in limited use [3, 24, 52, 74]. Various fusion inhibitors and RNA interference methods are also currently being studied for efficacy against RSV [3, 52]. RSV-specific antivirals currently under development target the F, G, N, or L proteins and although some are in clinical trials (Table 1), there are still no licensed therapeutics available.

Prevention and Control

Controlling and preventing RSV transmission can easily be done with proper sanitary practices such as hand washing, avoiding exposure to infected patients, and avoiding self-inoculation. Nosocomial spread of RSV can also be reduced by donning proper personal protective equipment, RSV infection surveillance, and isolating patients [3, 9, 52, 60]. Passive immunoprophylaxis is also an effective method in preventing RSV infection, primarily in premature infants. RSV Immune Globulin Intravenous (RSV-IGIV, RespiGam) was licensed in 1996. It is a polyclonal antibody prophylaxis composed of immunoglobulin purified from the sera of human donors that had high RSV-neutralizing activity. It is administered intravenously each month during the RSV season to high risk infants and young children. Although it reduced the frequency of hospitalization and the amount of time spent in the hospital, it is no longer commercially available for several reasons. RespiGam involved administering a high volume intravenously, could potentially interfere with other childhood vaccines due to the presence of antibodies specific to other

pathogens, and could not be used in children with cardiac comorbidities who were more susceptible to RSV infection [3, 20, 24, 39]. Palivizumab (Synagis) has taken the place of RSV-IGIV and was licensed in 1998. It is a humanized murine-based IgG1 monoclonal antibody that targets the F protein on both A and B subtypes. It has similar clinical efficacy as RSV-IGIV and is administered intramuscularly each month of the RSV season, but at smaller doses (15 mg/kg). However, it is an expensive treatment (currently about \$3,000 per vial) and reserved for high risk infants only [3, 60, 74]. A modified version of palivizumab, motavizumab (MEDI-524, Numax) has been produced and found to have high neutralizing activity *in vitro* and is more protective in murine models compared to palivizumab. Clinical trials with preterm infants further showed that it led to a greater reduction in RSV-related hospitalization. However, its association with increased hypersensitivity reactions, such as urticarial, has kept it from being licensed [60, 74].

Vaccines

Vaccines remain one of the best methods in preventing infection, but to this day there are still no licensed RSV-specific vaccines available. In the 1960s, an alum-adjuvanted formalin-inactivated RSV (FI-RSV) vaccine was developed and studied in a clinical trial of infants and toddlers. Although it was well tolerated, it was not protective. The vaccine led to disastrous results as the vaccinated subjects developed ERD after natural exposure to wild-type RSV. Of the test subjects, 80% required hospitalization and two died [25, 54]. Subsequent studies on the FI-RSV vaccine suggest that it induced an overwhelming Th2-biased immune response with poor neutralizing and CTL activities after wild-type infection which was attributed to poor TLR stimulation [17, 24, 57]. Persisting safety and efficacy concerns stemming from the results of the FI-RSV study is one of the main reasons there

are still no RSV vaccines commercially available. Over the years, many methods to produce a safe and protective RSV vaccine have been developed. Live-attenuated vaccines are showing some promise, but over- and under-attenuation must be avoided. Over-attenuation can result in a poorly immunogenic vaccine while under-attenuation can result in symptoms of respiratory disease. Cold-passaged temperature-sensitive (*cpts*) vaccines were developed to ideally restrict RSV replication to the nasal passage instead of the warmer lower respiratory tract. Results of a Phase 1 study using *cpts*-248/404 on 1- to 2-month old infants indicated that most of the infants showed no signs of RSV replication after 2 doses or ERD symptoms. However, signs of increased congestion hindered further development of the vaccine [7, 52, 80, 81]. An attenuated vaccine produced via reverse genetics called rA2cp248/404/1030ΔSH is currently in clinical Phase ½ Trials. This particular vaccine was generated by deleting the SH gene and introducing a temperature sensitive mutation in the L gene. It was well-tolerated by RSV-naïve infants and immunogenic and protective after a second dose, but less than half of infants who received two doses had a detectable NtAb response. Currently, rA2cp248/404/1030ΔSH as well as other live-attenuated vaccines are still being studied using other attenuating mutations [7, 37].

The use of vectors is another method used to produce RSV vaccines. An example of this is a recombinant chimeric human/bovine parainfluenza 3 (PIV3) expressing RSV F and/or G proteins that can potentially target both PIV and RSV, called MEDI-534. MEDI-534 is composed of the HN and F genes from human PIV3, the N, P, M, and L genes of bovine PIV3, and the RSV F gene. Although shown to induce protective immunity in the hamster

model, clinical studies in RSV seronegative infants showed that the immune response to RSV was inferior compared to that for PIV [5, 7, 24].

Subunit vaccines are also a promising approach, especially for the elderly population who are at high risk for RSV infection and disease. Purified F protein (PFP) vaccines have been evaluated in adults, older children, elderly patients, and pregnant women. Although the vaccines were well-tolerated and there were no signs of disease, the NtAb titers they induced were considered too low for further investigation [24, 27]. MEDI-7510 is a subunit vaccine composed of a glucopyranosyl lipid A (GLA)-adjuvanted post-fusion RSV F. GLA was added as a TLR agonist because previous studies have shown that it can enhance the murine antibody response to influenza vaccines [7, 44]. Novartis has developed a subunit vaccine composed of alum-adjuvanted post-fusion RSV F trimers. It induced NtAb and protective responses in the cotton rat model and the Phase 1 trial is expected to conclude by the end of 2016 [7, 73]. Novavax has engineered a RSV F nanoparticle vaccine using sf9 insect cells to produce post-fusion F protein. Cotton rat studies using this vaccine have shown that it can induce strong protective efficacy upon RSV challenge. In Phase 1 trials it was well tolerated and induced a dramatic increase in RSV F IgG. It is currently one of the few RSV vaccine candidates progressing to Phase 3 studies [7, 26, 63].

Virosomes are a relatively new method for vaccine development. Virosomes are a mechanism for vaccine and drug delivery in which the nucleocapsid is removed and the viral envelope is reconstituted to only contain proteins of interest. An example would be a virosomal RSV vaccine adjuvanted with monophosphoryl lipid A (MPLA) which acts as a TLR agonist. In the cotton rat model it was found that vaccination with these RSV-MPLA

virosomes induced high NtAb titers, an IFN- γ response similar to that induced by live virus, and protected the lower respiratory tract [36].

The use of virus-like particles (VLPs) is another promising method for developing a RSV vaccine and includes native and carrier VLPs. VLPs are genetically engineered complexes that express a repeating array of highly immunogenic protein antigens. When assembled, they morphologically resemble the real virus but do not contain any genetic material, making them safer to use [77]. Through reverse genetics, a recombinant Newcastle disease virus (NDV) expressing the RSV-F and -G proteins has been synthesized and used in a murine model. These carrier VLPs were essentially NDV VLPs except RSV F and G ectodomains were fused to the NDV F and HN proteins. The murine model study showed that immunization with these VLPs (without adjuvant) induced a robust anti-RSV F and anti-RSV G antibody and Th1-biased immune responses, as well as protecting the lungs of RSV-challenged animals [48]. Native VLPs, on the other hand, express the entire viral protein of interest rather than their ectodomains. These include RSV VLPs composed of the influenza M1 matrix protein and RSV F and/or G proteins. Results of a murine model study with these VLPs indicated that intramuscular immunizations of these VLPs induced NtAb antibody and IgG2a-dominant (suggesting a Th1-bias) responses [62]. Mammalian cell-derived native RSV VLPs composed of the F, G, and M proteins have also been developed. These have been found to be sufficiently immunoreactive and were able to induce a Th1-biased response. A cotton rat study with these VLPs further showed that they can induce potent NtAb and protective responses [78]. Table 2 lists some of the various RSV vaccines recently registered for clinical trials since 2008.

Summary

RSV is a Paramyxovirus found worldwide and is one of the leading viral agents associated with ALRI in infants and other high risk individuals. Of its 11 proteins, the F protein is of particular importance because it contains neutralizing epitopes that can be targeted by vaccines and antivirals. RSV immunopathogenesis is not definitively understood, but there are various viral, host, and environmental factors that can contribute to the severity of infection and disease. Since its discovery there have been many attempts to produce a vaccine against RSV, but due to persisting safety concerns (especially after the infamous FI-RSV vaccine developed in the 1960s) there are still no RSV-specific vaccines or therapeutics available.

Specific Aims and Hypotheses

Specific Aim 1. Demonstrate that immunization with MPLA-adjuvanted RSV VLPs induces a neutralizing antibody and Th1-biased response in the mouse model.

Hypothesis: Two doses of MPLA-adjuvanted RSV VLPs expressing the F, G, and M proteins can induce a neutralizing antibody and Th1-biased immune response in BALB/c mice.

Rationale: For this study, we used VLPs composed of RSV-F, -G, and -M proteins because the F and G proteins have been shown to be immunogenic and the M protein is required for morphogenesis. Six-week old female BALB/c mice were used. Previous studies have shown that immunization with VLPs expressing RSV F or RSV G can result in increased NtAb titers in the sera of murine models [53, 62, 78]. FI-RSV vaccination is also known to induce a Th2-biased immune response after wild-type RSV infection, which

contributes to most of the pathology, but it is possible to induce a Th1-biased response by using MPLA-adjuvanted vaccines [27, 36, 56, 78].

Experimental Plan: As shown in Fig. 3 groups of 6-week old female BALB/c mice ($n = 5$) were used. Group 1 (diluent group) were immunized with a TN buffer/sucrose mixture, Group 2 (RSV control) were infected with RSV at Day 0, Group 3 were immunized with MPLA-adjuvanted RSV VLPs, and Group 4 were immunized with non-adjuvanted RSV VLPs. Sera were collected at the indicated time points and used to determine NtAb titers (PRNT), IgG antibody titers (ELISA), and the Th1/Th2 response (ELISA and cytokine analysis) [59, 62, 77].

Specific Aim 2. Demonstrate that immunization with MPLA-adjuvanted RSV VLPs can confer protection against RSV infection in the mouse model.

Hypothesis: Immunization with MPLA-adjuvanted RSV VLPs expressing F, G, and M proteins protects the upper and lower respiratory tracts of BALB/c mice from RSV infection.

Rationale: Previous studies have shown that two doses of RSV VLPs were able to protect BALB/c mice from RSV lung infection [62]. Further studies using two doses of MPLA+alum-adjuvanted RSV VLPs protected cotton rats from lower respiratory tract infection showing the potential of using MPLA as a TLR agonist [78]. Based on these studies, using MPLA-adjuvanted RSV VLPs designed to express F, G, and M proteins to vaccinate BALB/c mice twice may be able to provide protection against infection in the upper and lower respiratory tracts. Furthermore, low viral titers in the nasal passage and lungs and minimal lung pathology may suggest a Th1-bias since the Th2-biased immune

response to RSV often leads to the excessive recruitment of immune cells to the site of infection [28, 56].

Experimental Plan: The left and right lungs collected on Day 46 will be separated, with the left lungs used to determine viral titers (plaque assays) and cytokine profiles (Luminex, to further characterize the Th1/Th2 response) in the lower respiratory tract and the right lungs used to look for evidence of pathology. The nasal washes will be used to determine viral titers (plaque assays) in the upper respiratory tract [70, 78, 79].

Significance and Innovation

There are many RSV vaccine candidates, however none have been licensed even though RSV remains a threat to high risk populations, especially infants. Our MPLA-adjuvanted RSV VLPs could be a promising method to prevent RSV infection. To our knowledge, this is the first time this vaccine formulation will be used to study immunogenicity and protective efficacy in the upper and lower respiratory tracts of a murine model.

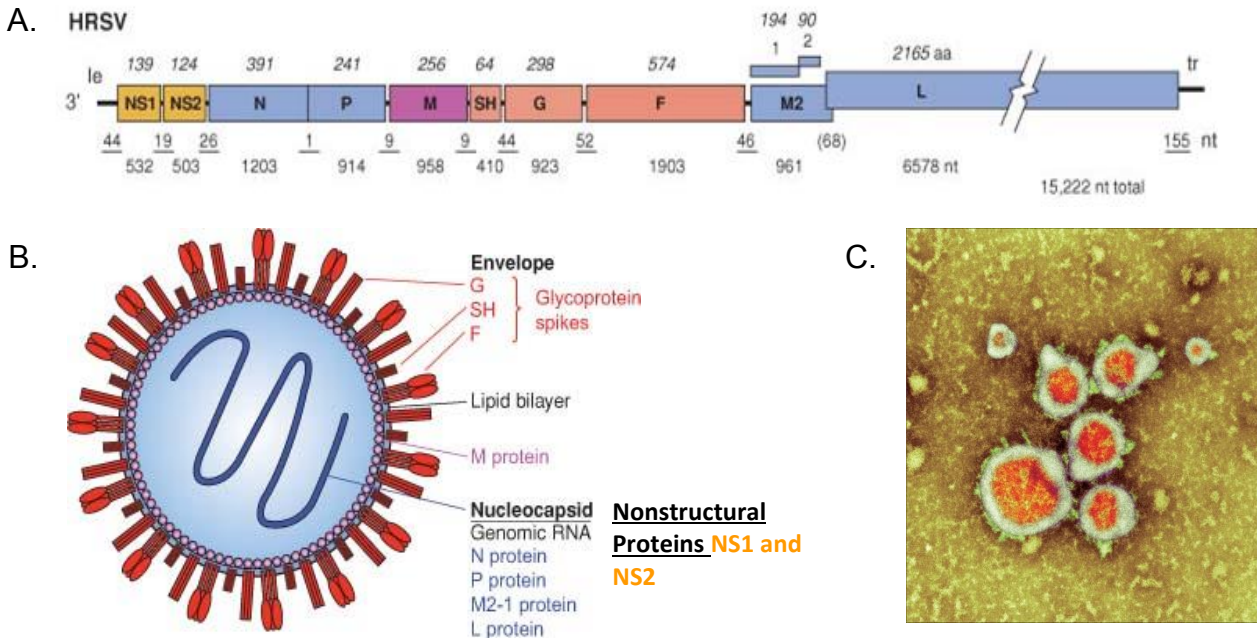


Fig. 1 RSV Genome and Structure

(A) RSV has a linear, non-segmented, single-stranded, positive-sense RNA genome approximately 15 kb in length. It contains 10 genes which encodes for 11 proteins: F, G, SH, M, N, P, M2-1, M2-2, L, NS1, and NS2. (B) The RSV virion is pleiomorphic and composed of a nucleocapsid within a lipid envelope. The lipid bilayer is derived from the infected host cell and expresses the F, G, and SH RSV surface proteins. (C) The colored transmission electron micrograph shows RSV particles containing the nucleocapsid (orange) within an envelope (white) expressing the surface glycoproteins (green). Images used with permission from Wolters Kluwer Health (A and B, Fields Virology, 2013) and Elsevier (C, Hall, C.B., 2010).

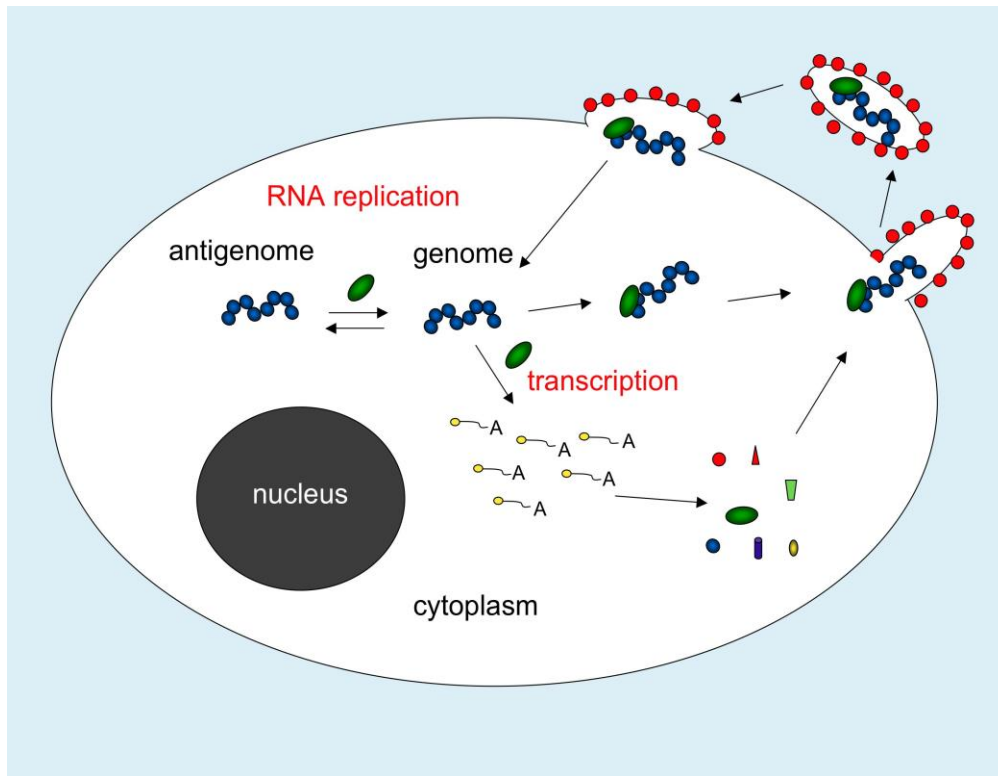


Fig. 2 RSV Replication Cycle

A schematic representation of the RSV replication cycle. Image used with permission from Dr. Rachel Fearn, Boston University, 2016.

Drug name	Clinical trial status	Manufacturer/ Institution	Experimental approach	Outcome
GS-5806	Phase 2b	Gilead	RSV entry inhibitor	Estimated completion June 2015
ALN-RSV01	Phase 2b (completed May 2012)	Ablynx	siRNA targeting the N protein	Reduced bronchiolitis obliterans post-RSV infection in lung transplant recipients.
ALS-008176	Phase 2a	Allos biopharma Inc.	Nucleoside analogue	Estimated completion August 2014
Danirixin	Phase 1	GlaxoSmithKline	CXCR2 antagonist. Inhibition of neutrophil activation	Estimated completion July 2014
RV568	Phase 1	Respivert Ltd	Kinase inhibitor	Completed 2011. Data not available
MDT-637	Phase 1	MicroDose Therapeutx, Inc	Fusion inhibitor	Commenced 2013. Data not available
ALX-0171	Phase 1	Ablynx	Nanobody	Commenced 2014 Data not available

Table 1 RSV-Specific Antivirals

A list of some of the RSV-specific antivirals recently registered in clinical trials since 2008.

Image used with permission from Dr. Ultan Power, Queen's University Belfast, 2015.

Drug name	Clinical trial status	Manufacturer/ Institution	Experimental approach	Outcome
<i>Vaccines</i>				
Live attenuated MEDI-559	Phase 1/2a	MedImmune LLC	Live attenuated	Increased rate of LRTIs in vaccine recipients, further study ongoing
RSV LID ΔM2-2	Phase 1	NIAID	Recombinant live attenuated RSV	Estimated completion May 2016
RSV cps2, Lot RSV#005A	Phase 1	MedImmune, NIAID	Recombinant live Attenuated RSV	Estimated completion May 2015
Chimeric/vectored RSV 001	Phase 1	Okairos	Adenovirus vector and an MVA vector encoding RSV antigens	Commenced May 2013 Outcome awaited
MEDI-534	Phase I	MedImmune LLC	Chimeric/vectored	Genetic variants within vaccine detected. Ongoing research.
<i>Nanoparticle</i>				
RSV F nanoparticle vaccine	Phase 2	Novavax	Recombinant RSV F protein particle	Commenced October 2013 Outcome awaited
<i>Subunit</i>				
MEDI 7510	Phase 1a	MedImmune LLC	RSV sF antigen + synthetic glucopyranosyl lipid A adjuvant	Estimated completion July 2015
RSV F subunit vaccine	Phase 1	Novartis vaccines	F subunit vaccine	Estimated completion September 2016
GSK3003891A	Phase 1	GlaxoSmithKline (GSK) Biologicals	Prefusion F subunit vaccine	Estimated completion March 2015

Table 2 RSV Vaccines

A list of some of the RSV vaccines recently registered in clinical trials since 2008. Image used with permission from Dr. Ultan Power, Queen's University Belfast, 2015.

CHAPTER 2

MATERIALS AND METHODS

Cells

Vero cells between passages 135 to 145 were used for all assays. They were maintained in T175 cell culture flasks in Dulbecco's Modified Eagle Medium (DMEM, Corning, Manassas, VA) with 10% fetal bovine serum (FBS, Atlanta Biologicals, Atlanta, GA) and 1% penicillin streptomycin (PenStrep, Life Technologies, Grand Island, NY).

Virus

RSV A2 strain was used for all in vitro assays and for infecting mice. Virus stock was prepared by growing Vero cells in T75 flasks up to ~70-90% confluency. Purified virus was added at MOI = 0.1. DMEM with 2% FBS and antibiotics. Cells were left to incubate until 90% cytopathic effects (CPE, ~2-4 days). Cells were scraped in the media and transferred to tubes for centrifugation at 2000 rpm in 4°C for 15 minutes. Remaining pellet was resuspended and sonicated using the long probe at setting 10 for 10 seconds on then 2 seconds off 20 times (Fisher Scientific Sonic Disembrator Model 100, Hampton, NH). Cells were centrifuged at 2000 rpm in 4°C for 10 minutes. Supernatant was transferred to a pre-chilled tube and 50% sucrose was added. After mixing, virus was aliquoted in snap-frozen, then stored in -80°C.

Adjuvants

Monophosphoryl lipid A (MPLA) derived from *S. minnesota* R595 (InvivoGen, San Diego, CA) was used.

Antibodies

Goat anti-RSV (Millipore, Temecula, CA) and donkey anti-goat IRDye 800 CW (Licor, Lincoln, NE) antibodies were used for PRNTs and plaque assays while goat anti-mouse IgG (H+L)-HRP, goat anti-mouse IgG1-HRP and goat anti-mouse IgG2a-HRP (SouthernBio, Birmingham, AL) were used for ELISAs.

VLPs

RSV VLPs were harvested, purified, and morphologically and immunologically verified as previously described by Walpita, P., et al. [78].

Western Blot

VLP composition was verified using a Western blot analysis. The purified VLPs were loaded into a 4-12% Bis-Tris Plus gel using 4x LDS sample buffer (Invitrogen, San Diego, CA) and ran for approximately 30 minutes at 200V. The proteins were transferred to a PVDF membrane for 3 hours at 60V in 4°C. The blot was blocked in Odyssey blocking buffer (Licor, San Diego, CA) for 1 hour at room temperature. The blot was then incubated overnight at 4°C with a 1:350 dilution of goat anti-RSV in Odyssey blocking buffer. After four 5-minute washes using phosphate buffered saline with 0.1% Tween 20 (PBST), the blot was incubated with a 1:15,000 dilution of IRDye 800CW donkey anti-goat IgG in Odyssey blocking buffer for 1 hour. After washing, the proteins were visualized using Odyssey infrared imager.

Immunogold Labelling

Purified RSV VLPs were adsorbed on to glow-discharged formvar-coated nickel grids (EM Sciences, Hatfield, PA). They were stained overnight with RSV-specific polyclonal antibodies diluted in 1% BSA in PBS, rinsed with 0.1% BSA in PBS, and stained with

colloidal gold-labeled secondary antibodies (EM Sciences, Hatfield, PA). They were then washed and negatively stained with 2% uranyl acetate.

Animals

Mouse studies were done with approval from the Institutional Animal Care and Use Committee (IACUC). Twenty 6-week old female BALB/c mice (Charles River, San Diego, CA), approximately 13-17 kg in body weight, were separated into 4 groups of 5 mice. Fig. 3 shows the vaccination schedule and experimental design. At Day 0, the diluent group (Group 1) was given 50 µl of a TN buffer/sucrose mixture, the RSV control group (Group 2) was intranasally given 10 µl/nares of 3×10^6 PFU/mL of RSV/A2, the VLP/MPLA group (Group 3) was given 50 µl of the MPLA-adjuvanted RSV VLPs, and the VLP group (Group 4) was given 50 µl of non-adjuvanted RSV VLPs. Sera were collected at the indicated time points. All injections were intramuscular and given at Days 0 and 21. At Day 42, Groups 1, 3, and 4 were intranasally challenged with 10 µl/nares of RSV. At Day 46, all mice were sacrificed using CO₂ inhalation so that nasal washes and lungs could be collected.

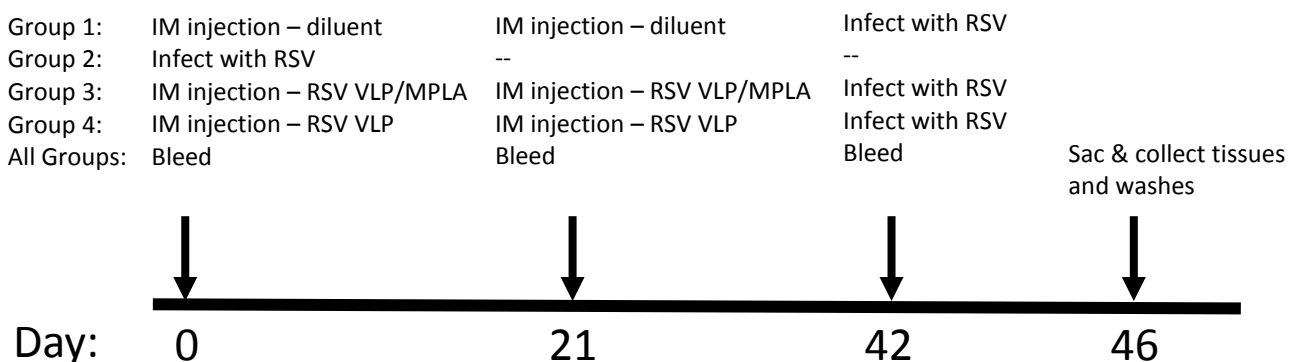


Fig. 3 Experimental Plan

Preparation of Nasal Washes and Tissues

Following euthanasia, nasal washes were harvested by disarticulating the jaw and removing the head. Then, 2 mL of Iscove's media (Invitrogen, San Diego, CA) with 15% FBS-MEM was injected through the nasal cavity and collected from the nares into a 5 mL tube. Each mouse nasal wash was separated into two 1 mL tubes then snap frozen for future use. The left and right lungs were harvested after euthanasia. The left lung was immediately placed in a lysing matrix tube containing 800 μ L of Iscove's media. It was then homogenized at 4.0 m/s for 20 seconds (MP Biomedical FastPrep-24, Santa Ana, CA) until no detectable large pieces of tissue were detectable. An additional 200 μ L of Iscove's media was added before it was centrifuged for 1 minute at 10,000 rpm at 23°C. The supernatant was separated into two 1 mL tubes and snap frozen for future use. For histopathology analysis, the right lung was separated from the left and 10% formalin (Sigma, St. Louis, MO) was perfused into the lung. Each inflated lung was dehydrated in ethanol and left in its own jar containing 10% formalin for fixation and later use in histopathology slides.

Plaque Reduction Neutralization Tests (PRNT)

Vero cells (1.3×10^5 cells) were seeded on 96-well plates and incubated overnight at 37°C and 5% CO₂. Sera were serially diluted 2-fold for a final volume of 25 μ L. Virus was diluted to 2000 PFU/mL in DMEM. The diluted virus and sera were incubated together for 1 hour at room temperature. DMEM from cell culture was removed and 50 μ L of the virus/sera mixture was added to each well, then incubated for 1 hour. The virus/sera mixture was removed, the wells were washed with PBS, 150 μ L of media was added to each well, and the plates were incubated for 48 hours at 37°C and 5% CO₂. The cells were fixed using

2% paraformaldehyde (EM Sciences, Hatfield, PA), washed with 1X PBS, permeabilized with 0.2% TritonX (Alfa Aesar, Ward Hill, MA), washed with 1X PBS, then dried and stored at 4°C until use. The wells were washed with PBS and 100 µl diluted primary antibody (goat α-RSV, 1:500) was added then left to incubate at room temperature for 2 hours. Primary antibody was discarded and the plates washed with PBST. 100 µl of diluted secondary antibody (donkey α-goat IRDye 800CW, 1:800) was added to each well, then left to incubate for 1 hour. Secondary antibody was discarded, the plates were washed with PBS, then scanned using Odyssey infrared imager.

Enzyme-linked Immunosorbent Assay (ELISA)

Vero cells were seeded on 96-well plates and incubated overnight at 37°C and 5% CO₂. The media was removed and virus was added at an MOI of 0.1 then incubated for 48 hours at 37°C and 5% CO₂. The cells were then fixed with 2% paraformaldehyde and permeabilized with 0.2% TritonX, then dried and stored at 4°C until use. The wells were blocked with 200 µl of 5% BSA in PBS and incubated overnight at 4°C. The blocking solution was decanted and 50 µl of serially diluted sera in 5% BSA was added to each well and incubated at 4°C overnight. The wells were decanted and washed with PBS. A 1:4,000 diluted secondary antibody (IgG, IgG1, or IgG2a) was added then plates were left to incubate for 1-2 hours at room temperature. The secondary antibody was decanted and the wells were washed with PBS. 100 µl of Ultra TMB (Thermo Fisher, Rockford, IL) was added to each well and incubated at room temperature for 15-30 minutes. 50 µl of the stop reaction (Thermo Fisher, Rockford, IL) reagent was added. Absorbance was measured at 405 nm using Victor X plate reader (PerkinElmer).

Cytokine Analysis

Cytokine concentrations for Day 46 sera were measured using a Luminex-based Th1/Th2 multiplex ProcartaPlex Cytokine Assay Kit (Affymetrix, Santa Clara, CA) which detected IFN- γ , IL-12p70, IL-4, IL-5, IL-6, and TNF- α .

Plaque Assay

Vero cells were seeded at 8×10^5 cells per well in 3 ml using 6-well plates, then incubated for 24 hours at 37°C and 5% CO₂. Media was removed from the 6-well plates so only ~200-300 μ l remained, then 200 μ l of the nasal wash or lung homogenate from each mouse was added, in duplicate. The plates were incubated at 37°C and 5% CO₂ for 1 hour. The wells were then overlaid with 3 ml of a 1:1 agarose/MEM mixture and incubated at 37°C and 5% CO₂ for 7 days. After 7 days, the cells were fixed using 2% paraformaldehyde, washed with 1X PBS, then permeabilized with 0.2% TritonX. The wells were washed with PBS and 1 mL diluted primary antibody (goat α -RSV, 1:500) was added to each well then left to incubate at room temperature for 2 hours. Primary antibody was discarded and the plates washed with 1X PBS/0.1% Tween. 1 mL of diluted secondary antibody (donkey α -goat IRDye 800CW, 1:800) was added to each well, then left to incubate for 1 hour. Secondary antibody was discarded, the plates were washed with PBS, then scanned using Odyssey infrared imager.

Histology

Right lungs were perfused with and stored in formalin immediately after collection. The formalin was later replaced with 70% ethanol to dehydrate the tissue. Slides were made by embedding fixed and dehydrated right lung tissue in paraffin wax and slicing 4 μ m thick sections. The sections were placed on glass slides and left to dry overnight. The tissue

slides were rehydrated then stained with hematoxylin and eosin. After the eosin stain, they were dehydrated again then observed under a microscope. Slides were evaluated for inflammation using a system similar to the one used by Smith et al. [70] and Prince et al. [61].

Statistical Analysis

The data was analyzed using GraphPad Prism 7. Statistical significance was determined using t-tests and 2-way ANOVA for multiple group comparisons. P-values <0.05 were considered statistically significant.

CHAPTER 3

RESULTS

Specific Aim 1: *Demonstrate that immunization with MPLA-adjuvanted RSV VLPs induces a neutralizing antibody and Th1-biased response in the mouse model.*

Verifying the presence and composition of RSV VLPs

We conducted a Western blot and used transmission electron microscopy (TEM) to verify the presence and composition of the RSV VLPs used in this study. Fig. 4 shows the Western blot results in which Lane 2 represents the RSV VLPs and Lane 3 is represents the cell control. The bands at ~150, ~100, and ~25 kDa show that the RSV VLPs successfully expressed the F, G, and M proteins, respectively. To determine immunoreactivity, immunogold labelling was used in combination with TEM. Fig. 5 shows two TEM examples of VLPs that reacted with RSV-specific polyclonal antibodies.

Determining the NtAb response using PRNT and IgG titers using ELISA

A high NtAb response is essential for an effective RSV vaccine and one of the main goals of previous studies was to induce high NtAb titers [43, 53, 62]. To determine the effect of using MPLA-adjuvanted RSV VLPs on inducing a NtAb response in BALB/c mice, we ran a PRNT analysis in duplicate. Serially diluted mouse sera were incubated with live RSV then added to Vero cells. The Vero cells were left to incubate for another 48 hours after which they were fixed and permeabilized. To visualize plaques, anti-RSV primary antibody was added followed by IRDye 800CW secondary antibody for labeling. Fig. 6 shows that immunization with adjuvanted VLPs resulted in higher NtAb titers (presented as the log₂ dilution of serum that reduces plaque formation by 50%) compared to immunization with the diluent and non-adjuvanted VLPs and RSV infection. The data

suggests a trend of increasing NtAb titers over time in the RSV control, VLP/MPLA, and VLP groups, with a 7.8 log₂ mean titer seen in the VLP/MPLA group, 6.33 log₂ in the VLP group, 5.2 log₂ in the RSV control group, and no change in the diluent group. The statistical analysis, however, indicates that there was no significant difference in titers between Days 21, 42, and 46 for the RSV control and VLP only groups. There was no significant difference between Days 42 and 46 in the VLP/MPLA group, but a statistical significance when comparing Day 21 titers with those from Days 42 and 46.

Total IgG titers were also measured to determine whether immunization could induce a sufficient IgG response. ELISAs were performed in duplicate to measure absorbance in OD. The antibody titers shown are the reciprocals of the highest serum dilution above the average OD value of the negative controls. As shown in Fig. 7 the data suggests a trend of increasing IgG titers for the VLP only group, but according to the statistical analysis there was no significant difference between the VLP/MPLA and VLP only groups at Days 21, 42, and 46.

Determining the Th1/Th2 response with ELISA and cytokine analysis

The 1960s study using FI-RSV resulted in the development of ERD in vaccinated infants after exposure to wild-type RSV [25, 54]. One of the main characteristics of ERD is a Th2-biased immune response so an effective RSV vaccine must dampen the Th2 response in favor of Th1. In this study, ELISAs were conducted in duplicate to determine the presence of IgG1 and IgG2a. The indirect association of IgG1 to the Th2 response and IgG2a to the Th1 response [6, 55, 62] was used as a guideline to evaluate whether VLP/MPLA immunization could induce a Th1-biased response. Because an enhanced Th1 response is important in an effective RSV vaccine, we looked at IgG1 and IgG2a levels on Days 21

and 46 to see whether immunization with VLP/MPLA could induce an enhanced IgG2a response. Statistical analysis was conducted by calculating a cutoff value based on (mean OD) + 3(standard deviation) of the negative controls, then using the cutoff values to determine IgG1 and IgG2a titers for each mouse in the VLP/MPLA and VLP groups at Days 21 and 46. The mean titers and p-values were calculated to determine whether there were significant differences between IgG1 and IgG2a titers at two time points for the two groups. Fig. 8A-B show that IgG1 and IgG2a levels for the VLP/MPLA group increased over time because OD values were sustained at higher dilutions at Day 46. Furthermore, Day 46 results show that IgG2a levels were higher compared to IgG1, suggesting a Th1-biased response. Meanwhile, the VLP group (Fig. 8C-D) not only had lower IgG1 and IgG2a levels compared to the VLP/MPLA group, but IgG1 levels were higher compared to IgG2a. This suggests a Th2-biased response. The mean titers from Table 3 indicates that there was a significant difference between IgG1 and IgG2a levels for the VLP/MPLA and VLP groups at Day 46, but not at Day 21. A comparison of IgG2a levels between the VLP/MPLA and VLP groups at Day 46 further indicates that IgG2a levels in the VLP/MPLA group was significantly higher compared to the VLP group (p-value ≤ 0.0014).

To further characterize the Th1/Th2 response, we conducted a cytokine analysis using a Luminex Multiplex assay to detect the presence of Th1-associated cytokines IFN- γ , IL-12p70, and TNF- α and Th2-associated cytokines IL-4 and IL-5 in Day 46 sera. We also looked for the presence IL-6, a cytokine associated with Th1 and Th2 differentiation [18]. Although there was some variation between animals, our results suggest that although IL-12p70 and TNF- α concentrations were low across all groups, IFN- γ concentrations (in

pg/mL) could be higher in VLP/MPLA-immunized animals compared to ones given VLPs alone (Fig. 9). However, the statistical analysis indicates that there is no significant difference in IFN- γ concentrations between the two groups. IL-4 and IL-6 concentrations were also low across all groups, but IL-5 levels were significantly (p-value = 0.0410) higher for the VLP group compared to the VLP/MPLA which emphasizes a Th2 bias response when MPLA is not present in the vaccine formulation. The mean IFN- γ concentration for the VLP/MPLA group seems to be higher than the mean IL-5 concentration, suggesting that although there were high concentrations of both in the sera the cytokine response may be slightly Th1-biased. This will require further investigation.

Specific Aim 2: *Demonstrate that immunization with MPLA-adjuvanted RSV VLPs can confer protection against RSV infection in the mouse model.*

Determining protective efficacy with plaque assays and histopathological analysis

An effective RSV vaccine should confer protection in the host by preventing infection and/or preventing the development of disease. The Th2-biased response associated with ERD is also characterized by evidence of inflammation in the lungs [1]. Although studies such as those by Smith G. et al. [70] and Kamphuis, T. et al. [36] use insect cell-derived nanoparticles and virosomes, respectively, as potential vaccines, they emphasized the importance of histology in evaluating a RSV vaccine's ability to protect the host from ERD-related immunopathology. We first compared viral titers between our four groups by performing plaque assays in duplicate to determine whether MPLA-adjuvanted RSV VLPs could prevent infection. Because RSV is restricted to the upper and lower respiratory

tracts, we specifically looked at viral titers in nasal washes and lung homogenates. Nasal washes or lung homogenates were added to Vero cells. After incubating for an hour an agarose/MEM mixture was overlaid on top of the cells and was left for a week. To visualize plaques, the overlay was removed and primary and secondary antibodies were added similar to the procedure for PRNT. As shown in Fig. 10 VLP/MPLA immunization was able to protect the mice from upper and lower respiratory infection, with almost complete protection in the nasal passage. VLP immunization was unable to confer complete protection in the nasal passage, resulting in an average of 26 PFU/mL of nasal wash, and the lungs, with an average of ~91 PFU/g of lung tissue. A t-test further indicated that there was a significant difference in nasal wash viral titers between the VLP/MPLA and VLP groups, but not in the lung homogenate viral titers.

The histopathological assessment following intranasal RSV challenge (Fig. 11) shows that the lungs collected from VLP only-immunized mice had inflammatory markers, particularly lymphocytic infiltration. The normal (Fig. 11A), RSV-infected at Day 0 (Fig. 11B), and the VLP/MPLA (Fig. 11D) mouse lungs showed no signs of inflammation. The particular VLP mouse lung shown in Fig. 11E had a NtAb response that followed the trend of VLP mean titers from Fig. 6. It also had total IgG, IgG1, and IgG2a titers that were consistent with the data shown in Fig. 7 and 8. Interestingly, it had one of the higher levels of IFN- γ and IL-5 expression in the serum (24.4 pg/mL and 21.02 pg/mL, respectively) and had one of the highest viral titers in the lungs and nasal wash (107 PFU/g and 65 PFU/mL, respectively) out of all animals. The VLP/MPLA-immunized mouse whose lung is shown in Fig. 11D had high NtAb, total IgG, IgG1, and IgG2a titers that increased after the first immunization just like the other animals from the same group. It also had the

highest serum IFN- γ concentration and had no detectable virus in the lungs or nasal wash. Eosinophils were also detected in some VLP only mice although they did not represent the majority of immune cells contributing to inflammation. Interestingly, lymphocytic infiltration was predominantly found near the bronchioles and the perivascular space, with very little to no signs of inflammation in the alveoli.

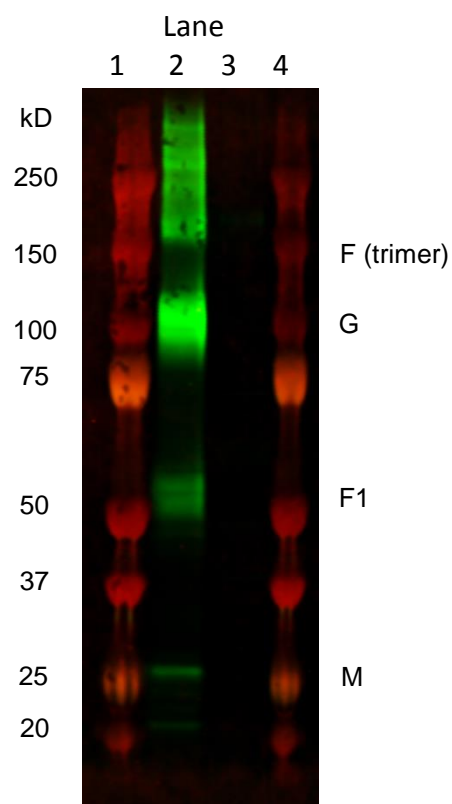


Fig. 4 Western Blot of RSV VLPs

RSV VLPs were used in this study. Lanes 1 and 4 represent the ladder while Lane 2 represents the RSV VLPs. Lane 3 is the cell control which does not react with the primary and secondary antibodies used for RSV VLPs. Western blotting confirmed the composition of these RSV VLPs by showing protein bands indicating the incorporation of the F, G, and M proteins.

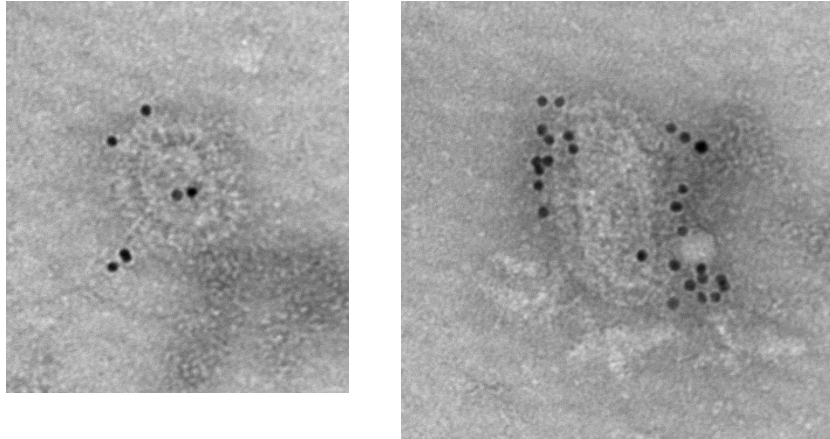


Fig. 5 Immunogold Labeling

Immunogold labeling confirmed the presence of these RSV VLPs by showing polyclonal RSV-stained immunogold particles bound to RSV VLPs.

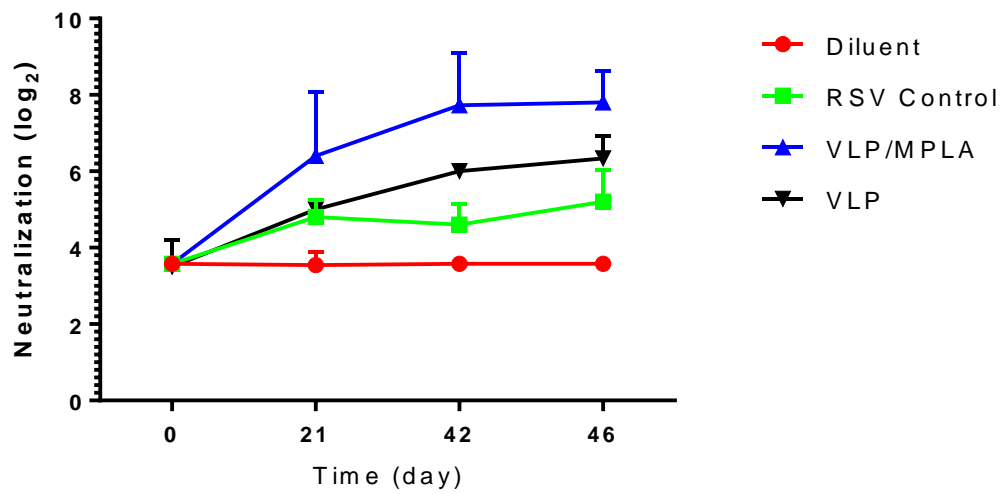


Fig. 6 NtAb Response

The average NtAb titers for each time point are presented as the log₂ serum dilution that resulted in a 50% plaque reduction ($n = 5$, mean \pm SD).

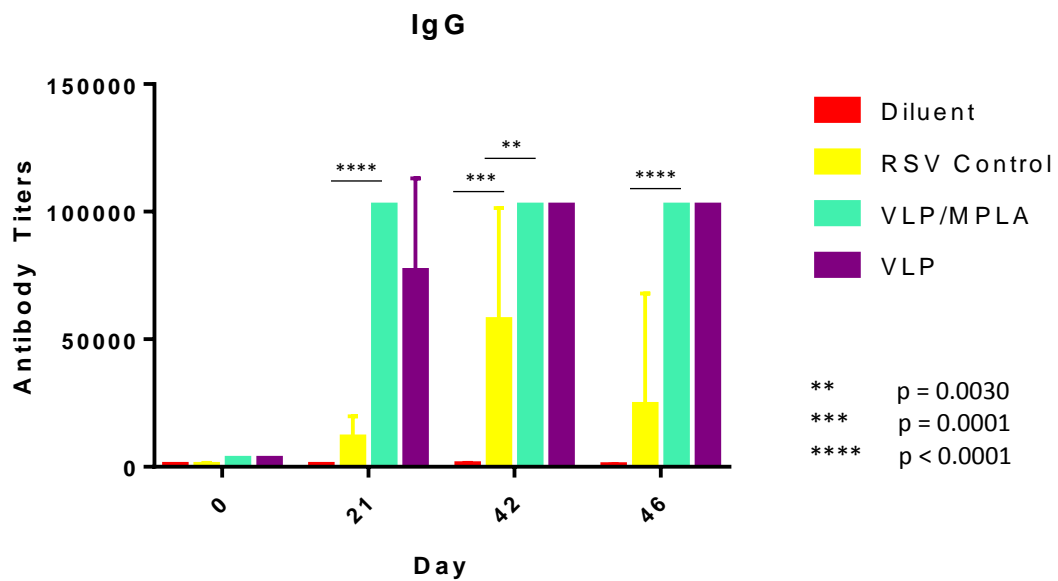


Fig. 7 IgG Response

Total IgG levels were determined using the ELISA method and measured in OD. The total IgG antibody titers shown here were the reciprocals of the highest serum dilutions above the average OD value of the negative control ($n = 5$, mean \pm SD).

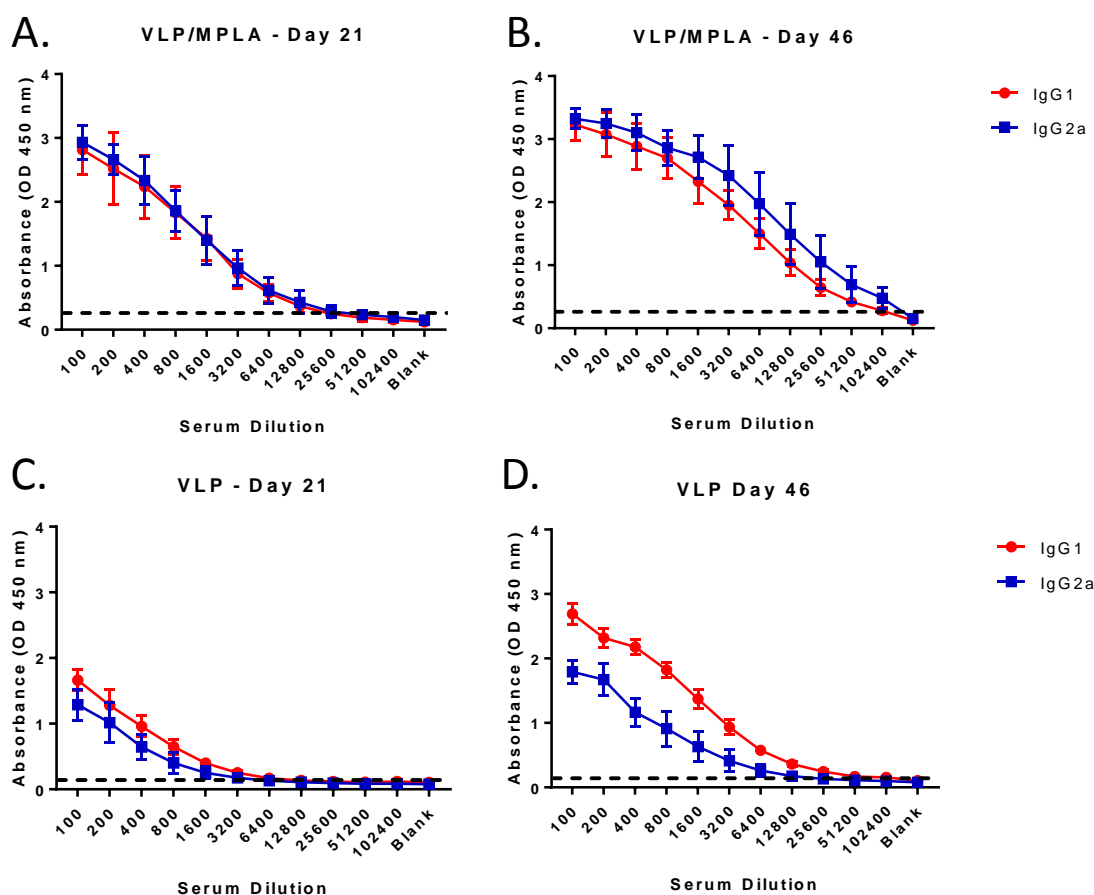


Fig. 8 Th1- or Th2-bias based on IgG1 and IgG2a Levels

A comparison of IgG1 and IgG2a absorbance at different time points ($n = 5$, mean \pm SD) for the VLP/MPLA (A-B) and VLP (C-D) groups using ELISA results shows an increase in IgG1 and IgG2a from Day 21 to Day 46. The higher IgG2a levels seen in the VLP/MPLA group, compared to the VLP group, suggests that the presence of MPLA can result in a Th1-biased response while its absence can lead to a Th2-biased response.

			Antibody Titers	Mean Titer ± SD	Significance (p-value < 0.05)
VLP/MPLA	Day 21	IgG1	12800	16640 ± 7680	NS (p-value = 0.3784)
			12800		
			12800		
			12800		
		IgG2a	32000		
			12800		
			25600		
			19200		
	Day 46	IgG1	6400	81920 ± 19157.29	Yes (p-value = 0.0381)
			51200		
			102400		
			76800		
		IgG2a	102400	102400	
			102400		
			102400		
			102400		
VLP	Day 21	IgG1	9600	9600 ± 2023.858	NS (p-value = 0.9212)
			12800		
			6400		
			9600		
		IgG2a	3200	4160 ± 2967.558	
			9600		
			4800		
			1600		
	Day 46	IgG1	1600	102400	Yes (p-value < 0.0001)
			102400		
			102400		
			102400		
		IgG2a	6400	20480 ± 15884.38	
			51200		
			19200		
			12800		

Table 3 Mean IgG1 and IgG2a Titers

Absorbance (OD) was measured using ELISA for each mouse in the VLP/MPLA and VLP groups ($n = 5$) at Days 21 and 46 to determine IgG1 and IgG2a levels. Cutoff values were calculated using mean OD + 3(standard deviation) of negative controls. Individual titers were the reciprocals of the highest serum dilution that intersected with the cutoff line. Mean titers and p-values were calculated to determine statistical significance. A

comparison of IgG2a between the VLP and VLP/MPLA groups at Day 46 indicates that IgG2a levels were significantly higher ($p\text{-value} \leq 0.0014$) for the VLP/MPLA group.

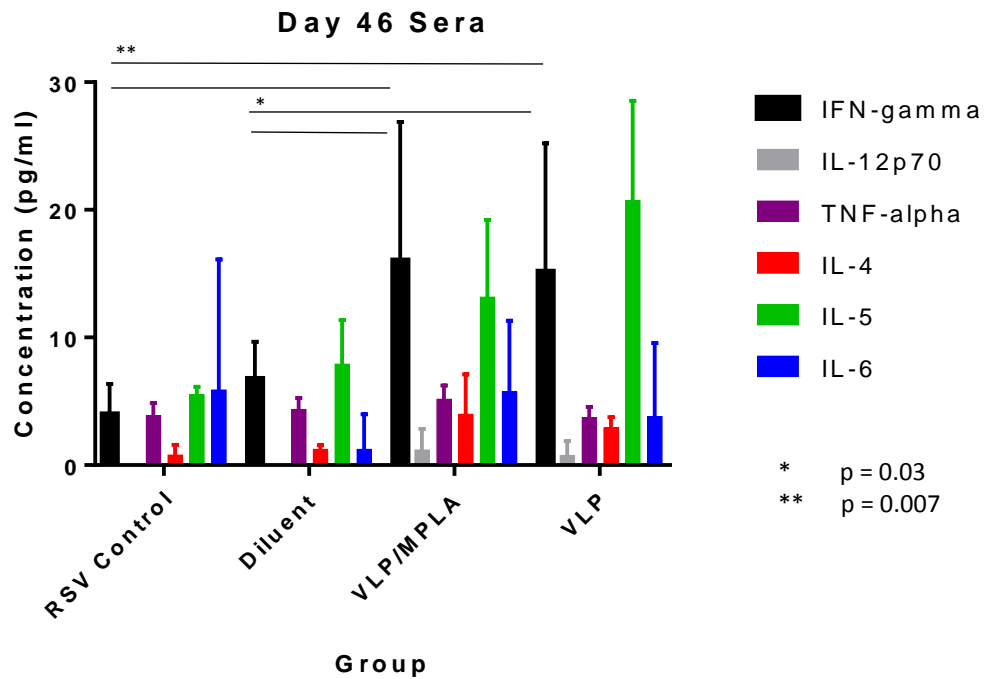


Fig. 9 Characterization of Th1 and Th2 Responses

Day 46 sera from all groups ($n = 5$, mean \pm SD) was used in a Luminex Multiplex assay specifically looking at concentrations of IFN- γ , IL-12p70, and TNF- α which are Th1-associated cytokines and IL-4 and IL-5 which are Th2-associated. We also looked at IL-6 concentrations, which is associated with both the Th1 and Th2 responses. Our results suggest a Th1-biased response.

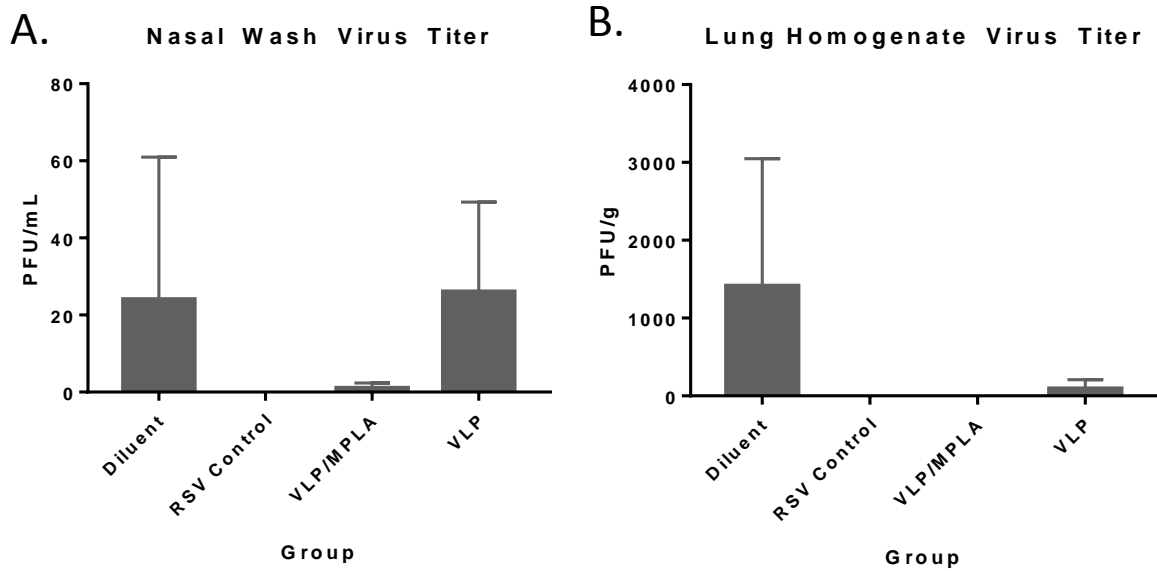


Fig. 10 Virus Titers in the Upper and Lower Respiratory Tracts

Using the plaque assay procedure described in Methods, RSV titers were measured for nasal washes (in PFU/mL of nasal wash) and lung homogenates (in PFU/g of lung tissue) at Day 46 ($n = 5$, mean \pm SD).

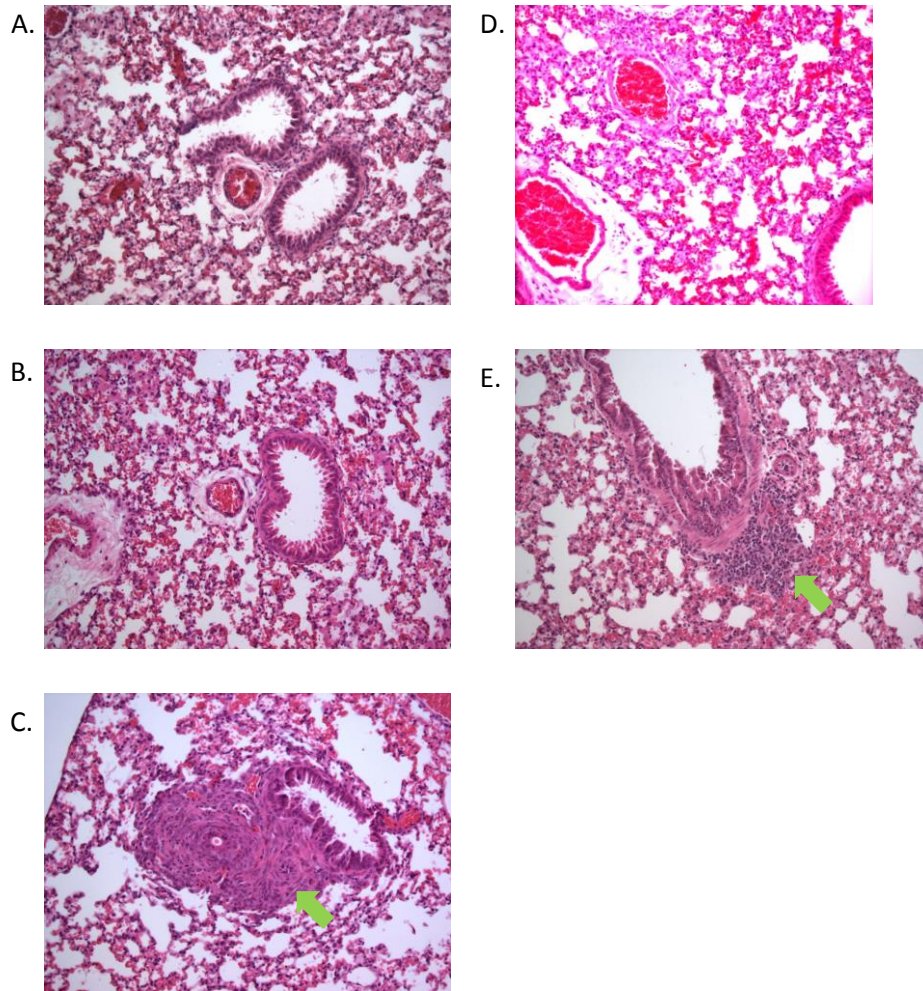


Fig. 11 Histopathological Assessment of Right Lungs Post-Challenge

Right lungs collected on Day 46, post-challenge with 3.0×10^6 PFU/mL of RSV, were fixed in formalin, embedded in paraffin, sliced in 4 μ m thick cross-sections, and stained with hematoxylin and eosin. (A) A negative control mouse, which was not immunized or challenged, exhibited normal histology. The RSV-infected (B) and VLP/MPLA-immunized mice (D) also showed normal histology, with no detectable signs of inflammation. The VLP- (E) and diluent (C)-immunized mice showed histopathological evidence of inflammation, predominantly lymphocytic infiltration, thus causing perivascularitis, vasculitis, and bronchiolitis (green arrows).

CHAPTER 4

SUMMARY AND DISCUSSION

Overview

Our objective in this study was to determine whether MPLA-adjuvanted RSV VLPs could (1) induce NtAb, RSV-specific IgG, and Th1-biased responses and (2) confer protection in a mouse model. We hypothesized that MPLA-adjuvanted RSV VLPs expressing the F, G, and M proteins can induce a NtAb, RSV-specific IgG, and Th1-biased responses and can protect the murine model from infection and disease. Our experimental design involved immunizing BALB/c mice with either adjuvanted or non-adjuvanted RSV VLPs twice. Our results suggest that two immunizations of MPLA-adjuvanted RSV VLPs can induce sufficient NtAb titers that are higher compared to immunization with the non-adjuvanted formulation. Our findings further indicate that it is possible for two immunizations with VLP/MPLA to enhance the Th1 response. We also found that, not only does VLP/MPLA immunization protect the respiratory tract from infection, it can also prevent the development of disease in the lungs.

Previous studies suggest that NtAbs are key correlates of protection for most paramyxoviruses, including RSV [62, 70, 77]. The induction of a NtAb response is important in many RSV vaccine studies because the likelihood of disease development is largely dependent on inhibiting viral replication [29]. The results of our NtAb response analysis using PRNT indicate that there is a trend of increasing NtAb titers after each immunization with either non-adjuvanted or adjuvanted VLPs. Although there is no statistical difference between Day 42 and 46 titers for either group, there seems to be a significant difference between Day 21 and Day 42/46 for the adjuvanted VLP group. In previous RSV vaccine studies, animals were typically sacrificed 4 days post-challenge [43, 53], but titers post-challenge have not always been considered. It is possible that the

4-day span between Days 42 and 46 is too short to notice a difference in NtAb titers. Statistical analysis does indicate that there is a significant difference between the VLP and VLP/MPLA groups at all time points except for Day 0 suggesting that adding MPLA to the VLP formulation enhances the NtAb response likely through its ability to stimulate TLR activity, just like the MPLA-adjuvanted virosomes developed by Kamphuis, T., et al. [36].

Using ELISAs, we further examined the presence of RSV-specific IgG in the serum. Fig. 7 shows that RSV VLPs with and without MPLA induced high titers of RSV-specific IgG, but there was no significant difference in titers between the two groups at Days 21, 42, and 46. In the Walpita, P., et al. study [78], the same VLPs were adjuvanted with an alum and MPLA mixture. The results indicated that there was a significant difference in cotton rat antibody titers between the adjuvanted and non-adjuvanted VLPs. Because alum is known to enhance the humoral immune response [13] perhaps a combination of the two adjuvants is necessary to achieve the high serum RSV-specific IgG titers that MPLA alone cannot.

FI-RSV immunization has been known to result in ERD, commonly characterized by an overwhelming Th2-biased response [25, 54]. The currently dominant theory is that this is due to the failure of FI-RSV to properly stimulate the TLR response [17]. To create a successful RSV vaccine, it is important to dampen the Th2 response in favor of Th1. Using ELISAs and a Th1/Th2 Multiplex cytokine analysis, we evaluated the ability of MPLA-adjuvanted RSV VLPs to induce a Th1-biased response. Our ELISAs specifically looked at IgG1 and IgG2a levels in the serum since IgG1 and IgG2a are indirectly associated with the Th2 and Th1 responses, respectively [62]. Our findings showed that

mice immunized with the adjuvanted formulation had similar levels of IgG2a compared to IgG1 after the first immunization (Day 21) and even higher after the second immunization (Day 46) (Fig. 8A-B). The significantly higher IgG2a titers compared to IgG1 at Day 46 further suggested that a second immunization of VLP/MPLA can enhance the Th1 response. The non-adjuvanted RSV VLP group, however, had inverse results in which IgG1 levels were significantly higher than IgG2a at Day 46, suggesting a Th2-biased response (Fig. 8C-D). These ELISA results highlight the importance of adding MPLA to the RSV VLP vaccine formulation. Our findings are supported by the insect cell-derived RSV VLP study by Quan et al. [62] in which the IgG2a:IgG1 ratios for both RSV-F and RSV-G VLPs were higher after the second immunization.

Because IgG1 and IgG2a titers are indirect measurements of the Th1/Th2 response, we also determined the presence of Th1- and Th2-associated cytokines in the serum post-challenge. Due to the large number of serum samples and the need to determine the concentrations of 6 cytokines for each mouse, we used a Multiplex bead-based assay. In the MPLA-adjuvanted RSV virosome study by Kamphuis et al. [36], they looked at the Th1 and Th2 cytokine levels in the lungs. They found that the MPLA-adjuvanted virosomes stimulated the expression of Th1-associated cytokine IFN- γ . Our findings showed that although some mice immunized with MPLA-adjuvanted RSV VLPs had heightened levels of IFN- γ , there was no statistical difference between IFN- γ levels between the adjuvanted and non-adjuvanted groups (Fig. 9). The non-adjuvanted vaccine formulation, however, resulted in a significant increase in IL-5 concentrations compared to the VLP/MPLA group, which is associated with the Th2 response, thus emphasizing that the lack of MPLA can lead to symptoms of ERD.

The fact that we did not see high concentrations of IFN- γ in the sera of VLP/MPLA-immunized mice could be due to more than the work of Th1 lymphocytes. IFN- γ is a key cytokine in viral clearance, produced mainly by CD4 Th1 and CD8 cytotoxic T lymphocytes (CTLs), and NK cells. Studies have shown that NK cell deficiency in a murine model can drive Th2-associated inflammation [35] and an increase in NK cell activity correlates with an increase in IFN- γ and CD8 T lymphocyte activity [28]. It is possible that NK cell and CD8 T lymphocyte activity, or lack thereof, may have hindered IFN- γ production in the VLP/MPLA mice and could be the subject of future investigations.

Upon investigating the ability of our MPLA-adjuvanted RSV VLPs to protect mice from RSV infection and disease, we found that not only do immunized mice exhibit low levels of virus in the upper and lower respiratory tract, but they also showed no evidence of developing clinical signs of inflammation in the lungs (Fig. 10-11). It is likely that the presence of NtAbs, RSV-specific serum IgG, and an enhanced Th1 response prevented the virus from causing disease and infection. Interestingly, despite the increased IL-5 concentrations detected in mice immunized with non-adjuvanted VLPs (Fig. 9), we did not see evidence of eosinophilia in the lungs. This may have more to do with limitations we encountered, as we will later discuss. We did find that the mice immunized with non-adjuvanted VLPs exhibited higher viral titers in the upper respiratory tract even though we found high antibody titers in the serum. Because it is unlikely for serum antibody to reach the nasal tissue [29], it is possible that the immunological mechanisms in the nasal passage were insufficient to combat the infection when there was poor TLR stimulation from the lack of MPLA. To our knowledge, this was the first time lung histopathology was examined in regard to the use of MPLA-adjuvanted RSV VLPs in a murine model.

The virus-like structure, the lack of genetic material, and the repetitive nature of viral proteins on the surface of VLPs have shown they are safe and immunogenic [42, 53, 62]. However, VLPs alone are sometimes incapable of inducing high antibody titers or are insufficient to confer complete protection. During these times, we can turn to adjuvants to improve immunity. MPLA alone and in combination with alum has been shown to be effective in murine models and is licensed for human use in Europe, particularly with the hepatitis B vaccine [2, 36, 78].

The use of MPLA-adjuvanted RSV VLPs is still in its infancy, but could have huge implications in pediatric pulmonary health. Because infants are one of the RSV vaccine target populations, a VLP-based vaccine could be used within the first few months of life, especially around the time when maternally-acquired antibodies begin to diminish. It might also be possible to use the vaccine on pregnant women to increase the amount of antibodies they pass on to the fetus. Altogether, our results suggest that the native conformation of our VLPs in addition to the presence of MPLA are safer than without MPLA and confer complete protection in the upper and lower respiratory tracts of the murine model, making them a promising RSV vaccine candidate.

Limitations and Future Studies

This study encountered several limitations, most of which were technical. In Specific Aim 1, we originally wanted to determine the presence of Th1- and Th2-associated cytokines in the lungs as done by Smith et al. [70] and Kamphuis et al. [36]. One of the main concerns with RSV infection is its potential to cause severe respiratory disease such as bronchiolitis and pneumonia. The increased presence of Th1-associated cytokines in the lungs of mice immunized with MPLA-adjuvanted RSV VLPs would indicate that the

enhanced Th1 response contributes to the protection of the lower respiratory tract. Upon attempting to use lung homogenates to conduct a Th1/Th2 cytokine analysis, we found very low, almost undetectable, concentrations of cytokines. When we collected lungs on Day 46, we immediately snap froze them for future use. Before conducting assays that required lung homogenates, we thawed the snap frozen lungs, homogenized them, then froze the remaining homogenates again for use in another assay. It is possible that the freeze/thaw process contributed to the inconsistencies we saw in the cytokine analysis of the lung homogenates.

We encountered similar issues when we conducted plaque assays to determine viral titers for Specific Aim 2. Typically, we diluted the nasal wash or lung homogenate sample. In doing so with our mouse lung and nasal wash samples, however, we found that diluting them produced very little to no plaques. Since this occurred even with the diluent group, in which we expected many plaques, we had to amend our protocol and use undiluted samples. Using PCR was a method that we considered to detect the presence of the virus in the samples, however PCR would not have allowed us to differentiate between active and inactive virus. Because it was important to determine whether MPLA-adjuvanted RSV VLPs could protect the upper and lower respiratory tracts from active infection, we chose to continue using plaque assays with undiluted samples.

In future studies, we plan on immediately homogenizing the lungs when they are collected, separating them into multiple smaller tubes, then snap freezing them all. This should reduce the number of freeze/thaw cycles and eliminate the possibility of inactivating the virus and lowering cytokine concentrations. As mentioned, there were high concentrations of IL-5 in mice immunized with non-adjuvanted VLPs, but we did not

see evidence of eosinophilia in the lungs. This may largely be due to the fact that we used sera to conduct the cytokine analysis instead of lung homogenates. Part of our future plans is to repeat the cytokine analysis using lung homogenates to determine whether non-adjuvanted VLPs do lead to high IL-5 concentrations and eosinophilia.

The ELISAs we conducted for this study also require further optimization. Our results do show that both the MPLA- and non-adjuvanted RSV VLPs led to high RSV-specific serum IgG titers. However, our statistical analysis indicated that there was no significant difference in antibody titer between the VLP and VLP/MPLA groups at Days 21, 42, and 46. The Day 21 data, in particular, may need to be further investigated due to the difference in mean titers.

Lastly, the number of time points and immunizations could be increased in future studies. Doing so would provide us with a better idea of the long-term effects on the overall mouse response and allow us to optimize the number and/or dosage of MPLA-adjuvanted RSV VLPs necessary to stimulate a sufficient NtAb and Th1-biased response.

Concluding Summary

In summary, our study regarding the safety and protective efficacy of MPLA-adjuvanted RSV VLPs showed that this vaccine formulation is capable of inducing sufficient NtAb, RSV-specific IgG, and Th1-biased immune responses. It also showed that the adjuvanted VLPs could confer protection in the upper and lower respiratory tracts. These findings not only highlight the importance of MPLA in enhancing the mouse immune response, but the potential of MPLA-adjuvanted RSV VLPs as a vaccine candidate as well. Despite the

promising results, future studies are still needed to determine the longevity of the protective response in a murine model and, eventually, its safety for human use.

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